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CENTRAL NERVOUS SYSTEM AXON REGENERATION using Rho Protein Inhibitors

Related Application Data

This application claims priority benefit of co-pending U.S. application serial number 60/055,268, filed on August 13, 1997.

Technical Field of the Invention

This invention relates to therapies for promoting central nervous system axon growth, including adenoviral-mediated gene therapy that results in a modification of growth cone signal transduction protein function. The treatment methods are particularly directed to recovery from acute or chronic spinal cord injury, traumatic brain injury, and white matter stroke.

Spinal cord function requires electrical conduction from one nerve cell to another through the extended axonal processes of these cells. After injury to the adult human spinal cord, these connections are interrupted, and the surviving nerve cells cannot communicate with one another to provide muscle control and sensation. Previous studies have indicated that the nerve cells are capable of re-extending their axons if given an appropriate environment. Unfortunately, the adult spinal cord is an inappropriate environment because inhibitory molecules are expressed by non-neuronal supporting cells. Thus, if the inhibitory influences can be overcome, then axonal regeneration and functional recovery may result.

Background of the Invention

Spinal cord injury is the prototypic example of a condition in which most axons are interrupted, but the vast majority of neuronal cell bodies remain intact. Although corticospinal pyramidal neurons in the cerebral cortex and large fiber sensory neurons in the dorsal root ganglia appear healthy, they cannot regenerate their injured axons. Chronic paralysis and anesthesia are the result of failed axonal regeneration. Lacunar white matter strokes and diffuse traumatic brain injury are similar in the sense that the axon itself is the major site of injury. In vertebrate systems, the ability of peripheral nervous system (PNS) but not central nervous system (CNS) axons to regenerate after injury is well known. Transplantation of sciatic nerve grafts into injured CNS tissue has demonstrated that CNS axons can regenerate through peripheral nerves (David and Aguayo, 1981).

Several CNS myelin-derived repulsive factors have now been identified. Antigenically related inhibitory activities of 35 kDa (NI-35) and 205 kDa into liposomes after SDS-PAGE have been reconstituted (Caroni and Schwab, 1988). NI-35 inhibits axonal extension and induces growth cone collapse (*id.*, and Bandtlow, *et al.*, 1993). An antibody to NI-35 promotes some axonal regeneration after spinal cord transection, demonstrating the physiological relevance of this inhibition (Schnell, *et al.*, 1994). Transplantation of olfactory ensheathing cells at the site of spinal cord injury can also promote a degree of axonal regeneration, presumably by substituting for the oligodendrocytes which normally produce inhibitory compounds (Li, *et al.*, 1997; Imaizumi, *et al.*, 1998; Mukhopadhyay, *et al.*, 1994).

In vitro, CNS myelin inhibition of neurite growth is also mediated in part by myelin associated glycoprotein (MAG; Mukhopadhyay, *et al.*, 1994; McKerracher, *et al.*, 1994). *In vivo*, MAG may or may not contribute to myelin inhibition of axonal regeneration (Bartsch, *et al.*, 1995; Schafer, *et al.*, 1996). If

the inhibitory effects of CNS myelin on axon outgrowth can be prevented *in vivo*, then increased recovery from spinal cord trauma and other instances of CNS axonal injury is likely to occur. Recent data on the micro-transplantation of embryonic neurons into adult CNS myelin tracts document some axonal extension within adult CNS myelin (Davies, *et al.*, 1997). The implication is that astrocytic scars as well as oligodendrocyte components contribute to the failure of adult CNS axonal regeneration. Repulsive factors are thought to act primarily on the specialized growth cone at the distal tip of the growing axon (Strittmatter, 1995; Strittmatter, 1996).

Neuronal growth cones possess the sensory apparatus to distinguish amongst innumerable potential pathways and targets during nervous system development and regeneration (for a review, see Strittmatter, 1995). Extracellular signals induce changes in the actin-based cytoskeleton of the growth cone and hence its morphology and motility. The molecular mechanisms whereby extracellular clues are transduced to cytoskeletal rearrangements are defined poorly.

The semaphorin/collapsin family of proteins has been recognized as one important negative regulator of axon outgrowth and terminal arborization (Luo, *et al.*, 1993; Kolodkin, *et al.*, 1992, 1993). Chick collapsin-1 induces growth cone collapse and a cessation of neurite outgrowth from at least a subset of DRG neurons (Raper and Kapfhammer, 1990; Luo, *et al.*, 1993). Insect semaphorins have a demonstrated *in vivo* role during axonal pathfinding and synaptic terminal branching (Kolodkin, *et al.*, 1992; Matthes, *et al.*, 1995). There are at least 7 vertebrate semaphorins identified and there may be as many as 20 members of this family (Puschel, *et al.*, 1995; Messersmith, *et al.*, 1995; Luo, *et al.*, 1995; Inagaki, *et al.*, 1995; Adams, *et al.*, 1996). A decrease in actin filaments after collapsin-1 application has been documented (Fan, *et al.*, 1993). The mechanisms whereby collapsin-1 binding to an unidentified transmembrane receptor triggers this depolymerization is unclear.

In non-neuronal cells, the rho subfamily of monomeric ras-related GTP-binding proteins have prominent effects on the actin-based cytoskeleton and on cell shape (Hall, 1990; 1994). In fibroblasts, rho activation has been linked to stress fiber ~~form—ation~~ formation and focal adhesions, rac1 activation with membrane ruffling and lamelipodia, and cdc42 activation with filopodial formation (Nobes and Hall, 1995). Single amino acid substitutions have been identified which produce constitutively active or dominant negative forms of each of these proteins. The C3 transferase from *C. botulinum* ADP-ribosylates rho specifically and inactivates the G protein.

The contribution of this class of G proteins to the regulation of neuronal growth cone motility has only recently come under investigation. In neuroblastoma cells, lysophosphatidic acid (LPA) or thrombin binding to heterotrimeric G protein-coupled receptors induces rapid neurite retraction (Jalink and Moolenaar, 1992; Jalink, *et al.*, 1994). The C3 transferase from *C. botulinum* has been shown to block the action of LPA, indicating that rho activation mediates LPA regulation of neurite length in ~~these~~ these cells (Jalink, *et al.*, 1994). A downstream target of activated rho has been identified as myosin light chain phosphorylase (Kimura, *et al.*, 1996), and an inhibitor of myosin light chain kinase, KT5926, also blocks LPA-induced neurite retraction (Jalink, *et al.*, 1994).

Further evidence for rho-related small G proteins in regulation of neurite outgrowth comes from studies expressing activated or dominant negative forms of these proteins *in vivo*. Alterations of rac1 activity, and to a lesser extent of cdc42 activity, lead to a failure in axonal extension from many neurons in the fly (Luo, *et al.*, 1994). Mice expressing constitutively active rac1 in cerebellar Purkinjie cells exhibit alterations in dendritic morphology (Luo, *et al.*, 1996).

The molecular mechanism whereby inhibitory (repulsive) molecules act on the distal tip of growing axons (the growth cone) are currently under study. In studies reported herein, it has been found that the GTP-binding rho protein is required for axon repulsion by a number of molecules (Jin and Strittmatter, 1997).

The inhibitory effects of CNS myelin on axonal growth in tissue culture are prevented by inhibition of the rho protein.

Summary of the Invention

It is an objective of the invention to utilize these findings to promote axon regeneration for the treatment of a variety of central nervous system disorders including acute or chronic spinal cord injury, traumatic brain injury, and white matter stroke.

These and other objectives are accomplished by the present invention, which provides methods for promoting central nervous system axon growth in patients in need of axon regeneration by administering to the patient an effective amount of at least one rho protein inhibitor such as rho, rac, cdc42 inhibitors, or mixtures of any of these. Rho protein inhibitors may be introduced mechanically to the axons or their non-neuronal support tissue, or introduced by administering replication-deficient adeno, adeno-associated, or herpes viruses that express inhibitors. In one embodiment the inhibitor is *C.botulinum* C3 exoenzyme; in another it is a chimeric *C. botulinum* C2/C3 inhibitor.

The invention correspondingly provides pharmaceutical compositions containing rho protein inhibitors for the treatment of central nervous system injuries using the methods disclosed herein. Also provided are screens that can be used to detect axon regenerative activity in panels of compounds by assaying for rho inhibitory activity.

Description of the Figures

Figures 1A and 1B show line graphs illustrating that collapsin-1-induced growth cone collapse is attenuated by KT5926 and PTX. (A) Two hours prior to the assay, the indicated concentrations of KT5926 were added to the DRG explant

culture medium. Low concentrations of KT5926 shifted the collapsin dose response curve to the right by a factor of 5. KT5926 had no direct effect on growth cone collapse in the absence of collapsin-1. The means from 4-6 separate experiments are shown. For each point, the SEM was less than 10% of the value shown. (B) Chick DRG explant cultures were pre-incubated for 3 hours in growth medium with the addition of 500 ng/ml pertussis holotoxin or with 500 ng/ml of oligomer B subfraction of pertussis toxin. Then, growth cone collapse was measured in the presence of the indicated concentrations of recombinant collapsin-1-His_6. While the oligomer B fraction had no effect, pertussis holotoxin decreased growth cone collapse at 200 pM collapsin-1 significantly ($p < 0.05$, Student's two-tailed t test). The average of five experiments with SEM is illustrated.

A and 2B, 2C and 2D

Figures 2 shows growth cone collapse and neurite outgrowth in DRG neurons triturated with rho subfamily proteins. (A) The protein preparations used for trituration were separated by SDS-PAGE and stained with Coomassie Blue. The migration of 45, 36, 25 and 21 kDa Mr standards is shown at the right. (B) DRG neurons were triturated with the indicated proteins at 5 mg/ml for rho family proteins and 0.1 mg/ml for C3 transferase. After 4 hours of culture, growth cone collapse was assessed with (gray bars) or without (solid bars) a 20 min exposure to 200 pM collapsin-His_6. The data are averages + SEM for 3-9 separate experiments. The values marked with an asterix are significantly different ($p < 0.05$, Student's two-tailed t test) from buffer-triturated cells under the same conditions. (C) DRG neurons were triturated with the indicated proteins and exposed to collapsin-1 as described in B. Actin was visualized by staining formalin-fixed cells with TRITC-phalloidin. Magnification, 500 X. (D) DRG neurons were triturated with the indicated proteins at 5 mg/ml for rho family proteins and 0.1 mg/ml for C3 transferase. After 2 hours of culture, neurons were exposed to 0 (solid bars) or 200 pM (gray bars) collapsin-His_6 for an additional 3 hours and then the average total neurite outgrowth per cell was determined (Goshima, *et al.*, 1995). The data are averages + SEM for 3-9 separate experiments. The values

marked with an asterix are significantly different ($p < 0.05$, Student's two-tailed t test) from buffer-triturated cells under the same conditions.

A, 3B, and 3C

Figures 3 shows rac1 in collapsin-1 regulation of growth cone motility. DRG neurons were triturated with buffer or various concentrations of the indicated G proteins. Growth cone collapse with or without a 20 minute exposure to collapsin-His_6 was determined as in Figure 2. The data are averages + SEM for 2-4 separate experiments. (A) Growth cone collapse after trituration with various concentrations of N17rac protein was determined with (○) or without (●) 200 pM collapsin. (B) DRG neurons were triturated with 0 or 2.5 mg/ml N17rac and 0 or 5 mg/ml of the following constitutively active G proteins: B is N17rac, C is N17rac+V14rho, D is N17rac+V12rac, and E is N17rac+V12cdc42; A is buffer. Growth cone collapse was determined in the absence (solid bars) or the presence (gray bars) of 200 pM collapsin-1. Note that V12 rac partially reverses the N17rac inhibition of collapsin-induced growth cone collapse. (C) After trituration with buffer (•), constitutively active V12rac (◇) or dominant negative N17rac (◆), growth cone collapse was quantitated for DRG neurons exposed to the indicated concentrations of collapsin.

A, 4B, and 4C

Figures 4 shows C3 transferase action on DRG neurons. DRG neurons were triturated and cultured as described in Figure 2. The data are averages + SEM for 2-4 separate experiments. (A) The indicated concentrations of C3 transferase were present during the trituration of DRG neurons. Growth cone collapse in the presence and absence of 200 pM collapsin-1 was determined as in Figure 2. (B) After trituration with buffer, 4 μg/ml C3 transferase, 5 mg/ml V14rho, or both proteins, neurons were exposed to 0 (gray bars) or 200 pM (solid bars) collapsin-His_6 and growth cone collapse was quantitated. In B (and C), A is buffer, B is C3, C is V14rho, D is C3+V14rho, E is C3+V12-rac, and E is C3+V12cdc42. (C) Average total neurite outgrowth per cell triturated as in B was determined after plating with (gray bars) or without (solid bars) the presence of 200 pM collapsin-His_6.

A and 5B

Figures 5 shows the effects of C3 transferase are not blocked by N17rac. DRG neurons were triturated with buffer, 5 mg/ml for N17rac, 0.1 mg/ml for C3

transferase or both proteins. The data are averages + SEM for 3-5 separate experiments. (A) Neurons were cultured for 4 hours and then growth cone collapse was assessed with (gray bars) or without (solid bars) a 20 min exposure to 200 pM collapsin-His_6. (B) The average total neurite outgrowth per cell for neurons triturated with the indicated proteins was determined after 4 hours after plating.

A, bB, and bC

Figures 6 shows that growth cone collapse by myelin or LPA is not blocked by N17rac. DRG neurons were triturated with the indicated proteins as in Figure 2. The data are averages + SEM for 3 separate experiments. (A) Neurons were cultured for 4 hours and growth cone collapse was assessed after a 30 minute exposure to buffer (solid bars), or CNS myelin extract (5 μg protein/ml, gray bars).

(B) After 2 hours of culture, neurons were exposed to 0 (solid bars) or 5 μg protein/ml CNS myelin extract (gray bars) for an additional 2 hours. The average total neurite outgrowth per cell was determined after 4 hours. (C) Neurons were cultured for 4 hours and growth cone collapse was assessed after a 30 minute exposure to buffer (solid bars), or LPA (1 μM, gray bars).

Figure 7 is a model drawing for rho/rac regulation of DRG growth cone function. Three states for DRG growth cones are classified by morphologic appearance, neurite outgrowth rate, rho activation state and rac1 activation.

Figure 8 schematically illustrates an adenovirus transfer vector map illustrating the major elements for expression of C3 exoenzyme or rac1 together with tau-EGFP. A polycistronic message is encoded: a Kozak translation initiation site and the coding sequence of C3 exoenzyme or of rac1 ending in a stop sequence is followed by a ribosomal reentry site and a second Kozak translation initiation site and the sequence for a marker protein. The marker consists of a fragment of tau protein for axonal targeting followed by an enhanced fluorescence variant of GFP.

Figure 9 is an immunoblot of adenovirus-directed expression of rac1 mutants. COS-7 cells were infected with recombinant adenoviruses expressing wild type rac1 (lane 1), V12 rac1 (lane 2), N17 rac1 (lane 3), or no rac1 protein (lane 4). Analysis of cells 24 hours after infection indicates that the low endogenous level of rac1 is greatly increased by recombinant adenovirus infection.

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Figures 10A and 10B histologically shows adenovirus-directed expression of C3 exoenzyme. COS-7 cells were infected with recombinant adenovirus expressing GFP (control, top panel) or C3 plus GFP (bottom panel). One day after infection, cells were fixed and actin filaments were visualized by rhodamine-phalloidin staining. The altered structure of the C3-expressing cells can be seen. Over 95% of cells were infected in the cultures.

Figures 11A and 11B shows that recombinant adenovirus expressing C3 prevents myelin-induced inhibition of neurite outgrowth. DRG neuronal cultures were infected with the C3/GFP adenovirus and then cultured for 4 days. Fluorescence microscopy demonstrates expression of the marker protein in cells with a neuronal phenotype (top panel). The cells were trypsinized and replaced without additions, with collapsin-1, or with extracts of CNS myelin. Note that neurite outgrowth is not decreased by the addition of these inhibitory factors (bottom panel). In control cultures, collapsin and CNS myelin decreased outgrowth by about 60%.

Figure 12 shows expression from the C3 recombinant adenovirus in rat cerebral cortex. The C3/EGFP adenovirus was injected into the cerebral cortex of 8 week old rats. Seven days later, the animals were sacrificed and the brains were examined by fluorescence microscopy. Note the intense cellular EGFP fluorescence at the injection site in the cerebral cortex. Similar results have been obtained with survival times up to 4 weeks. Similar expression is also obtained in DRG after local injection.

Detailed Description of the Invention

This invention is based upon the finding that rho protein inhibition promotes axonal regeneration after central nervous system injury by blocking the action of molecules in the injured spinal cord or brain which otherwise stymie functional recovery.

In the practice of the invention, axon regeneration is enhanced and growth promoted by administering an effective amount of at least one rho protein inhibitor to a patient in need of such treatment, *i.e.*, suffering from acute or chronic spinal cord injury, traumatic brain injury, white matter stroke, or other central nervous system injury that damaged axons and disrupted axonal tracts. By "rho protein inhibitor" is meant any inhibitor of rho protein function, analogues that bind to receptors, antibodies to the proteins or protein fragments, and the like. Mixtures of inhibitors can also be employed, as well as inhibitors of rho protein synthesis or stability. Rho protein inhibitors include any inhibitor of rho, rac, cdc42 or other protein in the GTP-binding subfamily. As used herein, "patients" include both animals and human beings; the invention has utility in both medical and veterinary applications.

Patients are treated by administering at least one inhibitor locally or systemically. Systemic administration can be via any method known in the art such as, for example, oral administration of losenges, tablets, capsules, granules, or other edible compositions; subcutaneous, intravenous, intramuscular, or intradermal administration, *e.g.*, by sterile injections; parenteral administration of fluids and the like. Typical systemic administrations involve the use of the inhibitor dispersed or solubilized in a pharmaceutically acceptable carrier.

Where administration is local, at least one inhibitor is typically introduced into the axons or their non-neuronal support tissue. Local administration of inhibitors includes, but is not limited to, mechanical introduction of the inhibitor

using any means such as injections, by perfusion or injection of the tissue with a composition containing the inhibitor in a pharmaceutically acceptable carrier, often in connection with ingredients that enhance penetration and uptake and/or the inhibitory activity, and by injection of recombinant viruses expressing inhibitors.

The last method is illustrated hereafter in Example 2. In this embodiment, *C. botulinum* C3 inhibitor, which inhibits rho proteins, is introduced intraneuronally to a patient using a replication-deficient adeno, adeno-associated, or herpes virus that express the C3. Recombinant adenoviruses, for example, have been utilized to direct neuronal expression of foreign genes over weeks to months with limited immunologic reaction in the CNS (Choi-Lumdberg, *et al.*, 1997). Adeno-associated viruses are employed in some embodiments because of their lower toxicity and long-term protein expression.

An alternate to the C3 inhibitor is a recombinant binary delivery system for the C3 exoenzyme, recently developed using the cell surface and binding components from the *C. botulinum* C2 toxin (Barth, *et al.*, 1998). The actin ADP-ribosylation activity was deleted from the C2 toxin and the C3 enzyme activity was substituted. This C3 chimeric protein is reported to enter non-neuronal cells at least 100-fold more efficiently than C3 exoenzyme itself. Use of this embodiment can involve direct injection of the molecule into the nervous system and achieve rho inhibition without the potential non-specific effects of viral injection.

The amount of inhibitor necessary to bring about the therapeutic treatment is not fixed *per se*, and is necessarily dependent on the concentration of ingredients in the composition administered in conjunction with a pharmaceutical carrier, adjunct compounds in the composition administered to enhance the inhibitory effect and/or penetration, and the age, weight, and clinical condition of the patient to be treated. Preferred compositions deliver the inhibitor in effective amounts without producing unacceptable toxicity to the patient. In addition to

penetration and uptake enhancers and/or inhibition activity enhancers, pharmaceutical compositions or formulations of the invention may also contain other carriers, adjuvants, stabilizers, preservatives, dispersing agents, and other agents conventional in the art having regard to the type of formulation in question.

The invention provides not only methods for stimulating axon regeneration and corresponding treatments for a variety of central nervous system injuries and pharmaceutical compositions used in the various therapies, but it also provides for screens that can be used to assay for rho protein inhibitory activity. In this aspect of the invention, panels of natural or synthetic compounds, including a variety of biological materials, are screened for potential in axon regenerative therapy using a rho protein inhibition assay such as rac1 inhibition. Screening tests may be quantitative or qualitative. Typical methods involve comparing inhibition observed by a panel of test compounds with control inhibition observed, for example, with *C. botulinum* C3 exoenzyme. The presence of inhibition indicates a potential agent for the stimulation of axon regeneration. Inhibitors identified by the screen can then be further tested, particularly for efficacy in either local and/or systemic administration.

Examples

The following examples are presented to further illustrate and explain the present invention and should not be taken as limiting in any regard.

Example 1

This example provides evidence that rac1 mediates collapsin-1-induced growth cone collapse. ~~Breifly,~~ Briefly collapsin-1/semaphorin III(D) inhibits axonal outgrowth by collapsing the neuronal growth cone lamelipodial and filopodial structures. Because growth cone collapse is associated with actin depolymerization, the small GTP-binding proteins of the rho subfamily was studied for its participation

in collapsin-1 signal transduction. Recombinant rho, rac1 and cdc42 proteins were triturated into embryonic chick DRG neurons. Constitutively active rac1 increases the proportion of collapsed growth cones, and dominant negative rac1 inhibits collapsin-1-induced growth cone collapse and collapsin-1 inhibition of neurite outgrowth. DRG neurons treated with dominant negative rac1 remain sensitive to myelin-induced growth cone collapse. Similar mutants of cdc42 do not alter growth cone structure, neurite elongation or collapsin sensitivity. Whereas the addition of activated rho has no effect, inhibition of rho with botulinum C3 transferase stimulates the outgrowth of DRG neurites. C3-treated growth cones exhibit little or no lamelipodial spreading and are minimally responsive to collapsin-1 and myelin. These data demonstrate a prominent role for rho and rac1 in modulating growth cone motility, and indicate that rac1 may mediate collapsin-1 action.

Materials and Methods

Preparation of proteins: G proteins, collapsin, myelin. Monomeric human G proteins and *C. botulinum* C3 transferase were produced in bacteria as GST fusion proteins and then treated with thrombin to remove the GST moiety (Nobes and Hall, 1995). Thrombin was removed from the samples by absorption to p-aminobenzamidine-agarose. The following derivatives were produced: wild type rhoA (rho), a constitutively active form of rhoA with gly at position 14 mutated to val (V14 rho), wild type rac1 (rac), a constitutively active form of rac1 with Gly at position 12 mutated to Val (V12 rac), a dominant negative form of rac1 with thr at position 17 mutated to Asn (N17rac), wild type cdc42 (cdc42), a constitutively active form of cdc42 with Gly at position 12 mutated to Val (V12 cdc42), a dominant negative form of cdc42 with Thr at position 17 mutated to Asn (N17cdc42), and the C3 exoenzyme from *C. botulinum* (C3). The rho and V14rho proteins contain a substitution of Asn at position 25 for Phe to enhance stability in *E. coli*.

Collapsin-His_6 was prepared as previously described (Goshima, *et al.*, 1995). Myelin fractions were prepared from bovine brain, and proteins extracted

with 2% octylglucoside were tested in growth cone collapse after removal of detergent by dialysis (Igarashi, *et al.*, 1992).

DRG culture conditions and trituration method. The preparation of chick E7 DRG explant and dissociated neuron cultures has been described previously (Strittmatter, *et al.*, 1994a; Goshima, *et al.*, 1995). For trituration experiments, neurons were suspended in 25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5 with 5 mg/ml rho subfamily proteins or with 0.1 mg/ml C3 transferase, and then passed 50 times through a Gilson P200 pipette tip (Strittmatter, *et al.*, 1994a; Goshima, *et al.*, 1995). After trituration, neurons were plated in 25 volumes of F12 medium with 10% FBS and 50 ng/ml 7S-NGF on a glass surface precoated sequentially with 100 μ/ml poly-L-lysine and 20 μ/ml laminin. For experiments with LPA, triturated neurons were transferred to serum-free medium (F12 medium with 1% fatty acid-free BSA and 50 ng/ml 7S-NGF) for 3 hours prior to the growth cone collapse assay.

Neurite outgrowth and growth cone collapse. For outgrowth assays, triturated cells were plated for 1.5-2 hours and then agents to be tested were added to the medium. After an additional 2-3 hours of incubation, the cells were fixed and total neurite length per neuron was measured for 75-150 cells (Strittmatter, *et al.*, 1994a; Goshima, *et al.*, 1995). The growth cone collapse assay for explant cultures has been described in detail (Raper and Kapfhammer, 1990; Strittmatter, *et al.*, 1994a; Goshima, *et al.*, 1995). For triturated cells, neurons were cultured for four hours prior to the addition of test compounds for 20-30 minutes. The fraction of collapsed growth cones was scored as for explant cultures.

Immunohistology. Dissociated chick E7 DRG neurons were cultured for 24 hours and then fixed with ice cold 4% paraformaldehyde, 20% sucrose in PBS for 30 minutes. Samples were then incubated with 4 μg/ml anti-rac1 mouse monclonal antibody directed against human rac1 (Upstate Biotechnology). In some cases, 1 mg/ml of rac1 protein was added to the incubation with antibody in order to demonstrate the specificity of the staining. Bound antibody was detected by the avidin-biotin-complex method (Vector Laboratories) with horseradish peroxidase enzyme and diaminobenzidine substrate as described (Goshima, *et al.*, 1995). The

addition of 1 mg ml recombinant rac1 protein to the primary antibody solution abolished all staining. Growth cones were also detectable by differential interference contrast observation.

Results

Comparison of collapsin-1 action with LPA and thrombin action. As a first step to assessing the role of small G proteins in collapsin action, the effect of readily available pharmacolgical agents on collapsin-1 action was compared to their effects on LPA and thrombin action. The myosin light chain kinase inhibitor, KT5926, blocks LPA-induced neurite retraction and also decreases the potency of recombinant collapsin-1 as a growth cone collapse factor (Figure 1A). A number of other agents had little or no effect on collapsin-1 action including tyrosine kinase inhibitors, protein kinase A inhibitors, voltage-sensitive Ca channel blockers and depolarization with KCl. The more general protein kinase inhibitor, staurosporine, and the protein kinase C activator, TPA, both induced growth cone collapse at concentrations below 10 nM, but their action was not synergistic with collapsin-1.

The actions of LPA and thrombin are mediated by receptors linked to heterotrimeric G proteins (Jalink, *et al.*, 1994). Whether recombinant collapsin-1 action also involves trimeric G protein activation was considered. Pertussis toxin (PTX) ADP-ribosylates the a subunit of heterotrimeric G proteins of the Go/i class and blocks their activation by receptors. Growth cone collapse by crude whole brain membrane extracts (BME, which contains collapsin-1) is blocked by PTX (Igarashi, *et al.*, 1992), but this is due to the cell surface binding properties of PTX rather than its modification of G proteins (Kindt and Lander, 1995). The isolated oligomer B fraction of PTX contains the cell surface binding domain but does not block purified recombinant collapsin-1-induced growth cone collapse (Figure 1B). Thus, the decrease in collapsin-1 potency by intact PTX suggests that collapsin-1 action involves heterotrimeric G protein action, strengthening the similarity with LPA and thrombin action. The failure of PTX blockade at higher collapsin-1 concentrations may be attributable to either PTX-insensitive G proteins

or to non-G protein-dependent mechanisms. Oligomer B blockade of BME action may reflect the inhibition of collapsing agents other than collapsin-1 in the crude extract.

Basal outgrowth in DRG neurons containing exogenous rho subfamily proteins. To modulate the activity of rho subfamily G proteins in DRG neurons, purified recombinant proteins were triturated with isolated neurons. Neurons were plated immediately after trituration; neurite extension and growth cone morphology were observed 2-5 hours later (Figure 2). All of the triturated proteins were greater than 95% pure (Figure 2A). Four hours after plating, neurons triturated with buffer are indistinguishable from cells which have not been triturated. None of the recombinant proteins affect the number of neurons which attach to the laminin-coated surface under these conditions. Of the proteins altering rho activity, only C3 transferase altered outgrowth. Neurite extension doubles after C3 transferase treatment (Figure 2D) and nearly all growth cones exhibit greatly reduced lamelipodial spreading (Figure 2B,C). These data raise the possibility that under basal conditions a significant fraction of rho is likely to be activated. Of the rac1 proteins, the constitutively active form increases the percentage of growth cones with a collapsed appearance (Figure 2B,C), and there is a slight trend towards decreased neurite extension which does not reach statistical significance (Figure 2D). These weak V12rac effects mimic the action of collapsin-1. The cdc42 proteins at the same concentration do not alter growth cone appearance or neurite extension.

Collapsin-1 sensitivity in DRG neurons containing rho subfamily proteins. Neurons triturated with rho family members were exposed to collapsin-1, and then growth cone morphology and neurite extension were examined. In control cultures, exposure to collapsin-1 for 30 minutes increases the percentage of collapsed growth cones from 15% to 70% (Figure 2B,C). Exposure to collapsin-1 during the interval from 2-5 hours after plating decreases the extent of outgrowth by 50% (Figure 2D). Collapsin-1-induced changes in growth cone collapse and neurite outgrowth are markedly attenuated in neurons treated with dominant negative N17rac (Figure 2B-D). In contrast, constitutively active

V12rac-treated and wild type rac-treated cells exhibit essentially normal responsiveness to collapsin-1. Trituration with cdc42 proteins or buffer does not alter collapsin-1 sensitivity. Similarly, wild-type and activated rho did not alter collapsin-1 action. However, the C3 transferase-treated neurons displaying increased neurite outgrowth are minimally sensitive to the inhibitory effects of collapsi n-1 (Figure 2D). The decreased lamelipodial morphology of growth cones in C3-treated cultures is only slightly enhanced by collapsin-1 (Figure 2B,C).

Characterization of rac1 effects in DRG neurons. The effect of dominant negative N17rac trituration is dependent on the dose of rac protein present during the trituration; concentrations in excess of 1 mg protein per ml are required to achieve greater than 50% inhibition of collapsin-1-induced growth cone collapse (Figure 3A). The specificity of N17rac action for endogenous rac1 pathways is suggested by the inactivity of dominant negative N17cdc42 (Figure 2B,D). Furthermore, the co-trituration of constitutively active V12rac, but not constitutively active V14rho or V12cdc42, partially reverses the N17rac inhibition of collapsin-1-induced growth cone collapse (Figure 3B).

After trituration with dominant negative N17rac, the collapsin-1 dose response curve for DRG growth cone collapse is shifted to the right by a factor of 15 (EC50 from 60 pM to 2 nM, Figure 3C). The residual weak effect of collapsin-1 as a growth cone collapse factor in N17rac-triturated cells may be due to incomplete rac1 blockade achieved by the trituration method, or to non-rac1-dependent collapsin-1-induced growth cone collapse mechanisms. As described above, trituration with constitutively active V12rac induces collapse of 20% of growth cones (Figure 2B). The dose response curve for collapsin-1-induced growth cone collapse is shifted to the left by a factor of 2 following trituration with constitutively active V12rac (EC50 from 60 pM to 30 pM, Figure 3C).

If rac1 is an endogenous modulator of collapsin-1-induced growth cone collapse, it must be present in the growth cone. Histologic staining for rac1 demonstrates that the protein is found in growth cones and is present in filopodial

structures at the very tip of the growth cone. Thus, the protein is in a position to mediate collapsin-1 action.

C3 action in DRG neurons. The ability of the C3 exoenzyme to specifically ADP-ribosylate rho in mammalian cells, including neuroblastoma cells, has been demonstrated previously (Jalink, *et al.*, 1994). The action of C3 transferase in DRG neurons depends on the dose of C3 exoenzyme present during the trituration, with as little as 1 μg/ml causing greater than 50% of DRG growth cones to collapse (Figure 4A). Although constitutively active V14rho does not alter basal growth cone collapse or outgrowth (Figure 2 B,D), trituration with this protein reverses the C3 effects on outgrowth and collapse (Figure 4B,C). Neither constitutively active V12rac nor V12cdc42 reverses C3 transferase action. Taken together, these data support the specificity of C3 transferase for rho inhibition after trituration into DRG neurons.

Dominant negative rac1 does not block the effects of rho inactivation. The decrease in growth cone area caused by C3 transferase treatment is associated with increased neurite extension, whereas that caused by collapsin-1 is associated with decreased extension. It was considered whether dominant negative rac1 could block the effects of rho inhibition by C3 transferase, as it blocks collapsin-1 action. When C3 transferase and N17rac are cotriturated, DRG neurites resemble C3-triturated neurites (Figure 5). Thus, modulation of neurite extension by rho is not mediated primarily through rac1. Rho may act in separate pathway(s) and/or function downstream of rac1 to regulate growth cone morphology and neurite extension.

Inhibitory effects of myelin are not mediated by rho family members. Components of CNS myelin have inhibitory influences on neurite regeneration and alter cultured DRG neuron morphology in a fashion similar to collapsin-1 (Bandtlow, *et al.*, 1993). Growth cone collapse after exposure to CNS myelin extract is not alt ered by trituration with N17rac (Figure 6A,B). This indicates that the $Ca^{+2}i$-dependent pathway utilized by inhibitory components of myelin (Bandtlow, *et al.*, 1993) is distinct from the rac1-dependent pathway utilized by collapsin-1. The rapidly growing, small growth cones present in C3-treated cultures are

insensitive to myelin (Figure 6A,B). Lysophosphatidic acid (LPA) induces collapse of a small fraction of DRG growth cones (Figure 6C). This fraction is not altered by N17rac, implying that LPA-induced collapse proceeds via a different pathway than collapsin-1-induced collapse.

Discussion

Rac1 mediates collapsin-1 action. Several lines of data from this study support the hypothesis that rac1 mediates collapsin-1 action in DRG neurons. Trituration of dominant negative N17rac nearly abolishes growth cone collapse by collapsin-1 and greatly reduces neurite outgrowth inhibition by collapsin-1. Other rho subfamily members do not have these effects. The presence of rac1 in the growth cone is consistent with a role in collapsin-1 signaling. Constitutively active V12rac weakly mimics collapsin-1 action. The small magnitude of V12rac action may be due to (1) the contribution of non-rac1 dependent mechanisms in collapsin-1-induced collapse, (2) the inefficiency of the trituration method or (3) desensitizing mechanisms occurring during the 3-5 hours after trituration. Although collapsin-1 action is inhibited by N17rac, the effect of other extracellular proteins which induce the same morphologic changes is not blocked by trituration with N17rac. This indicates that rac1 is specifically involved in collapsin-1 action and that the Ca^{+2}-mediated growth cone collapse induced by components of CNS myelin does not utilize this monomeric G protein.

Rho regulates neurite outgrowth, but is not altered by collapsin-1. Inhibition of rho with C3 transferase also alters the morphology of DRG neurons. This implies a significant level of rho activation in DRG growth cones under basal conditions. Further, the data suggest that rho activation may decrease outgrowth, but leads to greater growth cone spreading. In DRG neurons treated with a low dose of C3 to reduce rho activity, constitutively active V14rho does increase growth cone spreading and decrease neurite outgrowth. The decreased growth cone spreading and increased outgrowth rate of rho-inhibited neurons is only minimally modulated by collapsin-1. These effects distinguish rho action from rac1 activation and collapsin-1 addition. While it appears that rho exerts

different effects from rac1 and collapsin-1, growth cone morphology and motility may reflect additive rho and rac regulation. Although rho activation is downstream of rac1 activation in 3T3 fibroblasts (Nobes and Hall, 1995), this does not appear to be the case in DRG growth cones. Rho does not appear to be the primary mediator of collapsin-1 effects, but it may be a target for other DRG growth cone regulators, as suggested for LPA and thrombin (Jalink et al., 1994). The myosin light chain kinase inhibitor, KT5926, may counteract myosin l ight chain phosphorylase regulation by rho (Kimura, *et al.*, 1996). In so doing, this compound partially reproduces the C3 transferase effect and decreases collapsin-1 sensitivity.

Correlation of rho/rac1 activation with three states of DRG growth cone motility. The present study identifies three alternate states for DRG growth cones in culture (Figure 7). Under basal conditions, growth cones spread and advance at a moderate rate. Collapsin-1 decreases outgrowth rates and collapses growth cone lamelipodia and filopodia. Collapsin-1-induced alterations in growth cone behavior may be mediated by rac1 activation and are blunted by the presence of dominant negative N17rac. In contrast, inhibition of rho function by C3 transferase increases outgrowth rate while decreasing growth cone area. The basal state appears to be correlated with rho activation and rac1 inactivity.

Mechanism of rac1 activation: dbl proteins, G protein cascade, CRMP. The mechanism by which rac1 might be activated by extracellular collapsin-1 is unclear. In other cells types, proteins with domains homologous to the human Dbl act upstream of rac1 as guanine nucleotide exchange factors (Boguski and McCormick, 1993), but their presence in neuronal growth cones has not been studied. Receptors of several classes appear to be capable of activating rac1 in other cells, including receptor tyrosine kinases, serpentine receptors coupled to heterotrimeric G proteins and cytokine receptors of the TNF class. A central role for heterotrimeric G proteins in growth cone signal transduction is supported by a number of studies (Strittmatter, *et al.*, 1990; 1993; 1994b; 1995). Data presented here indicate that heterotrimeric G proteins (Figure 1B) may be involved in collapsin signaling. An intracellular family of neuronal proteins, CRMPs, has been

identified; these are required for collapsin action but their interaction with other members of this signaling pathway is not established (Goshima, *et al.*, 1995; Wang and Strittmatter, 1996). There are no data indicating that intracellular calcium ion levels are likely to mediate collapsin action.

Rac1 effectors in DRG neurons. Rac1 is capable of reorganizing the actin-based cytoskeleton in non-neuronal cells and of activating a number of protein kinases (Nobes and Hall, 1995; Hall, 1994; Cosco, *et al.*, 1995; Minden, *et al.*, 1995). Collapsin-1-induced changes in cell shape may be mediated by protein kinases such as PAK (Manser, *et al.*, 1994). After activation by rac1, such kinases are hypothesized to modulate cytoskeletal function.

Example 2

This example reports expression and biological activity of recombinant C3 adenovirus used for rho protein inhibition, and the *in vivo* modulation of neuronal rho protein activity.

As discussed above, the C3 exoenzyme from *C. botulinum* ADP-ribosylates rho specifically and inactivates this G protein. The contribution of this class of G proteins to the regulation of neuronal growth cone motility has only recently come under investigation. In neuroblastoma cells, lysophosphatidic acid induces rapid neurite retraction through a GPCR (Jalink, *et al.*, 1994). The C3 exoenzyme from *C. botulinum* has been shown to block the action of LPA, indicating that rho activation mediates LPA regulation of neurite length in these cells (Jalink, *et al.*, 1994). Injection of rho family proteins into neuroblastoma cells acutely alters growth cone morphology and axonal outgrowth (Kozma, *et al.*, 1997). Further evidence for rho-related small G proteins in regulation of neurite outgrowth comes from studies expressing activated or dominant negative forms of these proteins *in vivo*. Alterations of rac activity, by ~~expression~~ of constitutively active or dominant negative mutants, leads to a failure in axonal extension from many neurons in the fly (Luo, *et al.*, 1994). Mice expressing constitutively

active rac1 in cerebellar Purkinjie cells exhibit alterations in dendritic morphology (Luo, *et al.*, 1996).

Recombinant rho, rac1 and cdc42 proteins were triturated into embryonic chick DRG neurons in Example 1. The response of axons to collapsin-1 (semaphorin D/III), a prototypic diffusible axon repellent was examined. Constitutively active rac1 increases the proportion of collapsed growth cones, and dominant negative rac1 blocks collapsin-induced growth cone collapse and collapsin inhibition of neurite outgrowth. DRG neurons treated with dominant negative rac1 remain sensitive to myelin-induced growth cone collapse. Similar mutants of cdc42 do not alter growth cone structure, neurite elongation or collapsin sensitivity. Whereas the addition of activated rho has no effect, inhibition of rho with *botulinum* C3 exoenzyme stimulates the outgrowth of DRG neurites.

Neurite outgrowth increases to 150% of control levels after rho inhibition, and growth cones are reduced in size. C3-treated growth cones exhibit little or no lamelipodial spreading and are insensitive to collapsin or LPA. While CNS myelin extracts reduce outgrowth from control neurons by 50%, this inhibitory extract does not reduce outgrowth from C3-treated cultures.

In the Example 1 culture studies, purified protein is loaded into neurons by mechanical means. It does not enter neurons or ADP-ribosylate rho without trituration of individual cells. In order to deliver the enzyme intraneurally ~~initraneurally~~, recombinant adeno- and herpes viruses that express the C3 protein were derived. These vectors express C3 together with an enhanced fluorescent version of green fluorescent protein (EGFP, Clontech). Such vectors have allowed expression of other foreign proteins in neurons for 2 weeks (HSV, Carlezon, *et al.*, 1997) to 2 months (adeno, Choi-Lumbdberg, *et al.*, 1997) without toxic effects. The adenoviruses are El and E3 deleted, so that they are replication defective (He, *et al.*, 1998). The herpes virus preparations utilize the amplicon system; C3 and EGFP sequences were inserted into a plasmid containing the immediate early promotor 4/5 of HSV and an HSV packaging site. Recombinant virus preparations are obtained from a packaging cell line after sequential transfection with the amplicon

plasmid and infection with a immediate early gene 2 deletion mutant of HSV (Neve, *et al.*, 1997).

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Expression cassettes for the ~~protins~~ proteins of interest were constructed in a transfer vector, pQBI-AdBM5, with expression driven from the major late promoter of adenovirus (Figure 8; Massie, *et al.*, 1995). The linear transfer vector was co-transfected with the long arm of *Cla*I-cut E1/E3-deleted viral DNA into HEK 293 cells. Although the viruses are replication-defective, viral particles can be amplified in these cells because they are stably transfected to express the E1 protein element which is missing from replication-defective viruses. Viral stocks were plaque-purified twice, enriched by cesium chloride equilibrium centrifugation, and titered.

Such viral stocks were utilized to infect COS-7 kidney cells. Within 24 hours of infection, greater than 95% of the cells express the GFP marker protein as judged by the bright green fluorescence of living cells. The expression of the rac1 proteins was verified by immunoblot analysis (Figure 9). The expression of the C3 exoenzyme was documented indirectly by observing the change in actin filament staining in the virus-infected cells (Figure 10). The C3-expressing COS cells exhibit extensive protrusions without the lamelipodial spreading seen in control cultures.

The C3 virus was used to modulate rho function in DRG sensory neurons in culture. Five days after infection with virus an MOI (multiplicity of infection) of 10-100, essentially all neurons and non-neuronal cells in the DRG cultures express the GFP marker protein (Figure 11). Neurite outgrowth from cells infected with the C3 exoenzyme-expressing virus is insensitive to the inhibitory factors collapsin-1 and CNS myelin (Figure 11).

The C3-expressing virus was injected into the cerebral cortex of 8-week-old male rats, with the goal of infecting cortico-spinal pyramidal neurons. One week after injection large number of cells express the GFP marker (Figure 12).

The results show that the C3 viruses do infect sensory neurons in culture, direct expression of EGFP and render the neurons insensitive to semD and CNS myelin. It is clear that injection of the adenovirus into adult rat cerebral

cortex or DRG allows expression of the EGFP marker for at least 3 weeks. In preliminary studies, Nissl stained preparations there is no major cellular toxicity associated with viral injection.

The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

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The papers cited herein are expressly incorporated in their entireties by reference.

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